



Zooming in on the WNT/CTNNB1 Destruction Complex: Functional Mechanistic Details with Implications for Therapeutic Targeting

Saskia Madelon Ada de Man and Renée van Amerongen

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S. M. A. de Man · R. van Amerongen (✉)
Developmental, Stem Cell and Cancer Biology, Swammerdam Institute for Life Sciences,
University of Amsterdam, Amsterdam, The Netherlands
e-mail: r.vanamerongen@uva.nl

Abstract

WNT/CTNNB1 signaling is crucial for balancing cell proliferation and differentiation in all multicellular animals. CTNNB1 accumulation is the hallmark of WNT/CTNNB1 pathway activation and the key downstream event in both a physiological and an oncogenic context. In the absence of WNT stimulation, the cytoplasmic and nuclear levels of CTNNB1 are kept low because of its sequestration and phosphorylation by the so-called destruction complex, which targets CTNNB1 for proteasomal degradation. In the presence of WNT proteins, or as a result of oncogenic mutations, this process is impaired and CTNNB1 levels become elevated.

Here we discuss recent advances in our understanding of destruction complex activity and inactivation, focusing on the individual components and interactions that ultimately control CTNNB1 turnover (in the “WNT off” situation) and stabilization (in the “WNT on” situation). We especially highlight the insights gleaned from recent quantitative, image-based studies, which paint an unprecedentedly detailed picture of the dynamic events that control destruction protein complex composition and function. We argue that these mechanistic details may reveal new opportunities for therapeutic intervention and could result in the destruction complex re-emerging as a target for therapy in cancer.

Keywords

Destruction complex · Quantitative cell biology · Signalosome · WNT signaling

1 Introduction

All multicellular animals use WNT/CTNNB1 signaling for tissue patterning during embryonic development (Holstein 2012; Loh et al. 2016). This was first discovered in *Drosophila*, where loss-of-function mutations in *wingless* (*wg*, homologous to *WNT1* in mammals) lead to developmental defects in the embryo (Nusslein-Volhard and Wieschaus 1980). WNT/CTNNB1 signaling continues to be critical for tissue homeostasis and regeneration later in life, which is highlighted by the role that the mammalian WNT/CTNNB1 pathway plays in stem cell maintenance in multiple tissues, including the intestine, mammary gland, and skin (Barker et al. 2007; van Amerongen et al. 2012; Lim et al. 2013).

The fact that WNT/CTNNB1 signaling controls cell proliferation and differentiation in so many different contexts opens up possibilities – as well as challenges – for pharmacological intervention. For instance, the idea that WNT/CTNNB1 signaling could be boosted to promote regeneration and repair in aged or damaged human tissues has gained traction and, with the generation and design of specific agonists like potent WNT surrogates, now also promises to be within reach (Janda et al. 2017; Chen et al. 2020). Conversely, in cancer, where hyperactive WNT/CTNNB1 signaling is frequently observed, therapeutic targeting is aimed at blocking or inhibiting the pathway.

The first link between WNT/CTNNB1 signaling and oncogenesis came from a retroviral insertional mutagenesis study, where integration of the mouse mammary tumor virus (MMTV) in the *int-1* locus led to tumor formation (Nusse and Varmus 1982; Nusse et al. 1984). Shortly thereafter, this locus was shown to harbor a gene homologous to *wg*, and its murine counterpart was given the name *Wnt1* (Rijsewijk et al. 1987; Nusse et al. 1991). The importance of WNT/CTNNB1 signaling in human cancer became more clear when the Adenomatous Polyposis Coli (APC) protein was found to be part of the signal transduction cascade (Rubinfeld et al. 1993; Su et al. 1993). Truncations in APC had previously been shown to underlie Familial Adenomatous Polyposis (FAP), a condition ultimately leading to colon cancer. Hyperactivation of the WNT/CTNNB1 pathway through similar genetic mutations in APC or, other signaling components, as well as via non-genetic mechanisms are frequent events in many cancer types (Polakis 2000; Zhan et al. 2017; Nusse and Clevers 2017; Wiese et al. 2018). The pleiotropic involvement of WNT/CTNNB1 signaling in cancer makes it an attractive target for therapy.

WNT proteins (the name being an amalgamation of *wg* and *int-1*) activate signaling through Frizzled (FZD) and LDL Receptor Related Protein 5 and 6 (LRP5/6) transmembrane proteins. The basic signal transduction mechanism of the WNT/CTNNB1 pathway has been established for many years, and is excellently described and depicted elsewhere (e.g., Nusse and Clevers 2017). Briefly, in the absence of WNT proteins (“WNT off” situation), the central effector, Catenin beta-1 or β -catenin (CTNNB1), is targeted for degradation by the so-called destruction complex. In the presence of WNT proteins (“WNT on” situation), degradation is impaired and the levels of CTNNB1 rise. This is accompanied by a switch from nuclear exclusion to nuclear enrichment of CTNNB1 (Kafri et al. 2016; de Man et al. 2021). In the nucleus, CTNNB1 binds to the “WNT enhanceosome,” which contains Transcription factor 7, 7-like 1 and 7-like 2/Lymphoid enhancer-binding factor 1 (jointly referred to as TCF/LEF) transcription factors and many other proteins, to drive gene transcription (Behrens et al. 1996; Molenaar et al. 1996; Fiedler et al. 2015; van Tienen et al. 2017; Gammons and Bienz 2018).

It has long been appreciated that inactivation of the destruction complex is central to WNT/CTNNB1 pathway activation, but the exact underlying biochemical details remain incompletely understood (Tortelote et al. 2017). The developments in quantitative cell biology and functional imaging approaches of the last decade now not only allow these biochemical events to be measured with high precision, sensitivity and subcellular resolution, but they also are starting to reveal the dynamic details of the processes involved.

2 WNT Off: Destruction Complex Function

2.1 Partners, Interactions, and Levels

The multiprotein destruction complex consists of scaffolds Axin-1/Axin-2 (jointly referred to as AXIN) and APC, and kinases Casein Kinase 1 Alpha (CSNK1A1) and Glycogen Synthase Kinase 3 (GSK3). CSNK1A1 primes CTNNB1 at S45 for subsequent phosphorylation at T41, S37, and S33 by GSK3 (Amit et al. 2002; Liu et al. 2002). This generates a binding motif for Beta-Transducing Repeat Containing E3 Ubiquitin Protein Ligase (BTRC), which ubiquitinates CTNNB1, targeting it for proteasomal degradation (Aberle et al. 1997; Latres et al. 1999).

Notably, only AXIN has been reported to directly bind all other destruction complex components, indicating it acts as the central scaffold (Fig. 1a). AXIN has been referred to as having a “beads on a string” structure, in which several structured domains, including its N-terminal Regulators of G protein Signaling (RGS) domain (which mediates APC interaction) and its C-terminal Domain present in Dishevelled and Axin (DAX) domain (which mediates self-oligomerization), are interspaced with intrinsically disordered regions (Bienz 2014). Interactions with the kinases and CTNNB1 all occur via these intrinsically disordered regions. The net effect of these interactions is a proximity-based increase in affinity of CSNK1A1 and GSK3 for CTNNB1, thereby stimulating CTNNB1 phosphorylation and eventually its degradation. Although it was initially proposed that AXIN increases the phosphorylation rate of CTNNB1 by GSK3 over 10,000-fold (Dajani et al. 2003), recent work suggests that the direct increase is more modest (only two- to three-fold), as measured *in vitro* (Gavagan et al. 2020). Interestingly, this same study showed that when GSK3 substrates other than CTNNB1 were also present, AXIN1 non-specifically blocks GSK3B from binding and phosphorylating these other targets, while specifically rescuing the binding and phosphorylation CTNNB1. Thereby, AXIN1 could preferentially promote GSK3B-mediated CTNNB1 phosphorylation in more crowded environments such as the cytoplasm (Gavagan et al. 2020).

As AXIN can bind all destruction complex components, this raises the question why there is a need for an additional scaffold, APC. The APC protein binds AXIN through its three Ser-Ala-Met-Pro (SAMP) repeats as well as its armadillo repeat (ARM) region (Roberts et al. 2011; Pronobis et al. 2015). In addition, APC has three 15 amino-acid repeats and six 20 amino-acid repeats that bind CTNNB1 with different affinity. It has been well established that APC is required for proper destruction complex function (Mendoza-Topaz et al. 2011; Pronobis et al. 2017), as also underlined by the fact that APC truncations lead to the high levels of WNT/CTNNB1 signaling that can drive oncogenic transformation (Fodde et al. 1999). Multiple explanations exist as to why and how APC is required for CTNNB1 degradation. First, it was proposed that APC prevents CTNNB1 from undergoing dephosphorylation and thereby helps shuttle it toward BTRC mediated ubiquitination (Su et al. 2008). Second, APC might also be required to release CTNNB1 from AXIN in order to undergo proteasomal degradation (Pronobis

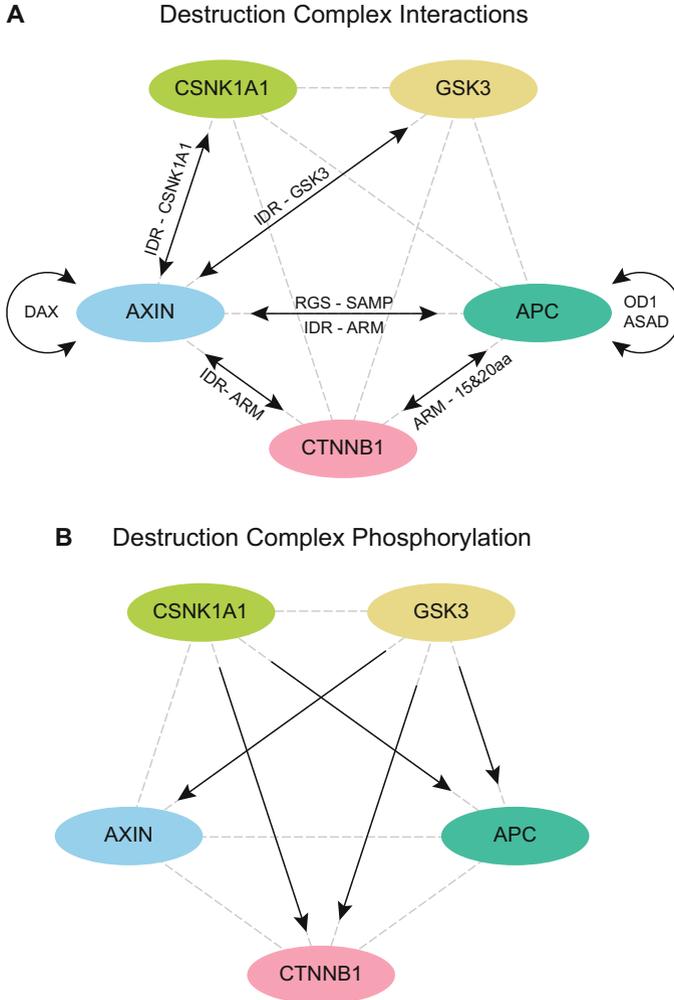


Fig. 1 Destruction complex interactions. **(a)** Physical Interactions between the core destruction complex partners. Protein domains involved in interactions for *AXIN*: IDR = intrinsically disordered region, RGS = Regulator of G protein Signaling domain, DAX = AXIN DIX domain, *APC*: 15&20aa = 15 and 20 amino-acid repeats, SAMP = Ser Ala Met Pro motifs, OD1 = Oligomerization Domain 1, ASAD = APC Self Association Domain, *CTNNB1*: ARM = armadillo repeats. **(b)** Phosphorylation within the destruction complex. In addition to *CTNNB1* CSNK1A1 also phosphorylates APC, and GSK3 phosphorylates *CTNNB1*, APC, and AXIN

et al. 2015). Finally, APC likely stabilizes the destruction complex by physically bridging AXIN filaments and by promoting AXIN phosphorylation (Yamulla et al. 2014; Pronobis et al. 2015; Tacchelly-Benites et al. 2018).

Phosphorylation of both scaffold proteins stabilizes the destruction complex, leading to more efficient degradation of *CTNNB1* (Pronobis et al. 2015). These

phosphorylation events appear to be mediated by the destruction complex kinases themselves (Fig. 1b). For instance, CSNK1A1 has been shown to phosphorylate APC at several residues (Jiang et al. 2018). In addition, GSK3 mediates both APC and AXIN phosphorylation (Rubinfeld et al. 1996; Yamamoto et al. 1999; Ha et al. 2004; Tacchelly-Benites et al. 2018). Further identification of the phosphorylated residues and their effect on destruction complex composition and dynamics will possibly identify crucial regulatory mechanisms of these proteins.

AXIN has long been assumed to be the rate-limiting component for destruction complex formation and function. This notion was based on measurements in *Xenopus* egg extracts where AXIN was determined to be present at very low levels of only 0.02 nM (Lee et al. 2003). In mammalian cell lines, however, AXIN1 levels were found to be substantially higher, averaging 20–150 nM (Tan et al. 2012). In contrast, APC levels were found to be lower, in the range of 4–24 nM (Tan et al. 2012). An independent study also concluded that APC, rather than AXIN1, was rate limiting for destruction complex formation (Kitazawa et al. 2017). If true, this could have profound implications: Knowing which components are limiting tells us something about the most essential interactions we might want to target pharmacologically. From a more fundamental perspective, it also informs our thinking about the molecular signal transduction mechanism.

2.2 Destruction Complex Oligomerization and Multivalency

Both AXIN and APC can interact with themselves (Fig. 1a). AXIN oligomerizes through its C-terminal DAX domain (Hsu et al. 1999; Sakanaka and Williams 1999). DAX domains undergo head-to-tail interactions, thereby forming longer filaments (Schwarz-Romond et al. 2007a). APC also oligomerizes, which in the case of destruction complex formation is mediated through one or more N-terminal domains (Joslyn et al. 1993; Kunttas-Tatli et al. 2014). Self-oligomerization of both AXIN and APC is generally thought to be required for proper destruction complex function, as illustrated by the reduced capacity of DAX domain AXIN mutants and APC deletions lacking the N-terminal APC self-association domain (ASAD) oligomerization domain to target CTNNB1 for degradation (Fiedler et al. 2011; Kunttas-Tatli et al. 2014; Pronobis et al. 2017). At the same time, a subtle (~four-fold) increase in AXIN levels has been reported to be able to partially compensate for the loss of a functional DAX domain in *Drosophila* embryos (Peterson-Nedry et al. 2008), which might be explained by multiple sites of interaction between the different destruction complex components and CTNNB1, which offer alternative modes for crosslinking to ensure proper function.

When overexpressed, AXIN can form large puncta that recruit APC and other destruction complex components (Cliffe et al. 2003; Faux et al. 2008; Kunttas-Tatli et al. 2014; Pronobis et al. 2015; Schaefer et al. 2020). These cytoplasmic puncta formed by the destruction complex partners have been shown to be highly dynamic, as measured by Fluorescence Recovery After Photobleaching (FRAP) (Schwarz-Romond et al. 2007b; Pronobis et al. 2015). There is some evidence that these large

dynamic structures also form at (near) endogenous levels (Fagotto et al. 1999; Faux et al. 2008; Mendoza-Topaz et al. 2011; Pronobis et al. 2015; Thorvaldsen et al. 2015; Schaefer et al. 2018). In *Drosophila*, AXIN puncta have been estimated to contain somewhere between tens to hundreds of molecules (Schaefer et al. 2018). The DAX-DAX interaction affinity is estimated to be in the low micromolar range (Kan et al. 2020). Based on this, it was concluded that at endogenous AXIN1 levels (around ~150 nM in HEK293 cells as measured by Tan et al.) oligomerization based on DAX-DAX interaction alone was unlikely to occur spontaneously. In agreement with the proposed function of APC to crosslink AXIN filaments, additional clustering by APC might be sufficient to induce AXIN polymerization at endogenous levels. Although yet to be confirmed by others, one intriguing new finding is that these destruction complex puncta can coincide with nascent *CTNNB1* mRNA, thereby directly targeting newly synthesized CTNNB1 for degradation (Chouaib et al. 2020).

In light of the above, what then is the precise identity of the destruction complex? Does it have a fixed size and stoichiometry or do larger and smaller varieties exist within the cell depending on the local concentration of AXIN and APC? And, related to this, how many CTNNB1 molecules can be captured by any one of these complexes? The destruction complex is generally thought to have high multivalency given that (1) both AXIN and APC bind CTNNB1, (2) APC has multiple binding sites for both CTNNB1 and AXIN, and (3) APC and AXIN oligomerization further increase the number of potential binding sites for CTNNB1. Whether all these binding sites are occupied or even accessible in a 3D complex, where steric hindrance comes into play, remains to be uncovered.

3 WNT On: Inactivation of the Destruction Complex

The precise mechanism of WNT/CTNNB1 pathway activation remains open to discussion even after 40 years of research. In any case, it requires inactivation of the cytoplasmic destruction complex. This, in turn, is ultimately controlled by the binding of WNT proteins to its cognate receptors. WNT binding induces a series of events, including receptor protein dimerization, Dishevelled (DVL) recruitment and LRP5/6 phosphorylation, ultimately generating the so-called WNT signalosome that subsequently inhibits the destruction complex (Fig. 2) (DeBruine et al. 2017b). Resolved protein structures and imaging studies that capture these events have revealed new details of how the WNT signal is initially transduced at the level of the plasma membrane – including the first insight into stoichiometric composition and quantified dynamics of signalosome formation.

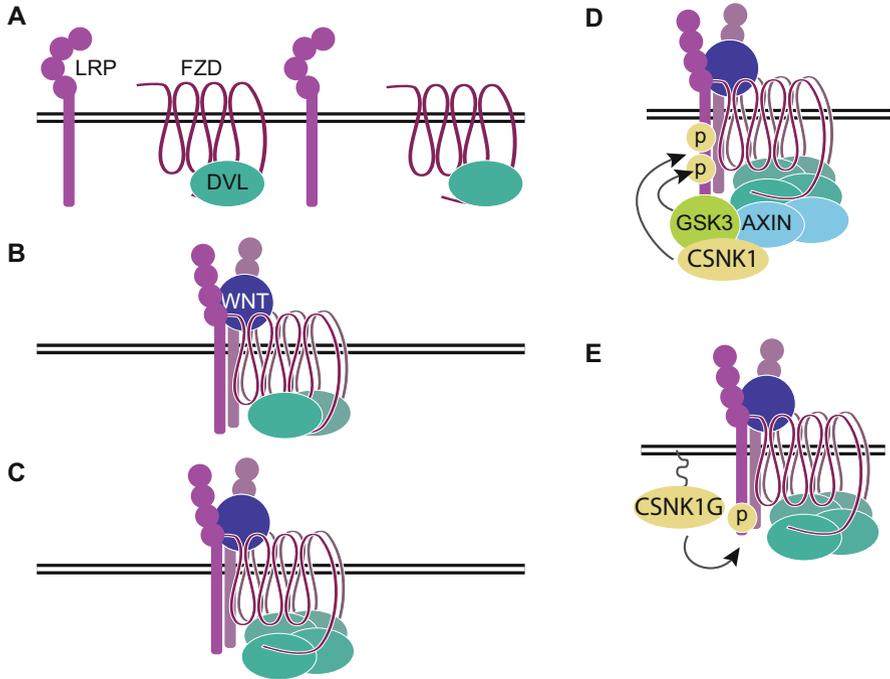


Fig. 2 WNT signalosome formation. **(a)** In the absence of WNT/CTNNB1 signaling, FZD and LRP5/6 proteins are already present at the membrane. DVL associates with FZD as a monomer. **(b)** Binding of a WNT protein drives homodimerization of FZD and heterodimerization of FZD-LRP5/6 proteins and conformational changes to the extracellular domains of LRP5/6. **(c)** Receptor dimerization induces DVL dimerization through DEP domain swapping and further oligomerizes through the DVL's DIX domain. **(d)** DVL oligomerization recruits AXIN to the receptor complex. Co-translocation of GSK3 drives phosphorylation of PPP(S/T)P motifs in LRP5/6 by GSK3 and subsequent phosphorylation of adjacent χ (S/T) motif by CSNK1A1/D/E. **(e)** CSNK1G phosphorylates LRP5/6 at two additional clusters surrounding the most C-terminal PPP(S/T)P motif. **(d)** and **(e)** complete the process of signalosome formation

3.1 Signalosome Formation

3.1.1 WNT Protein Binding to FZD and LRP5/6

Due to their covalent lipid modification, WNT proteins typically do not diffuse far in the aqueous extracellular environment. Instead, they tend to associate with the cell surface (McGough et al. 2020). The lipid moiety does more than limiting the spread of WNT, however, as it is also involved in WNT-FZD binding. The crystal structure of *Xenopus* WNT8 in complex with the cysteine-rich domain (CRD) of mouse FZD₈ revealed that the palmitoleic acid of WNT inserts itself into a groove on the FZD protein to help stabilize the WNT-FZD interaction (Janda et al. 2012). This binding event induces the formation of FZD dimers or tetramers as elegantly shown by Bioluminescence Resonance Energy Transfer (BRET) assays and further confirmed by protein crystallography (Nile et al. 2017; DeBruine et al. 2017a; Hirai et al. 2019).

Receptor dimerization has been demonstrated for multiple WNT/FZD combinations, suggesting that it may be a common mechanism. Of note, FZD dimers may already exist prior to WNT protein binding (Gerlach et al. 2014; Petersen et al. 2017). Using Fluorescence Cross Correlation Spectroscopy (FCCS), it was further shown that WNT5A binding first causes brief dissociation of FZD₆ dimers, before inducing reassociation (Petersen et al. 2017). This study illustrates the extent of detail and temporal resolution that can be obtained by such image-based techniques, which will ultimately be necessary to further elucidate the precise sequence of signal transduction events and the stochastic variation therein.

In addition to promoting FZD di- or tetramerization, WNT binding also induces FZD-LRP5/6 heterodimerization. In fact, heterodimerization alone is sufficient to induce downstream signaling (Cong et al. 2004; Janda et al. 2017). Recently, it was shown that a tetravalent antibody, which brings together two FZD and two LRP5/6 molecules, induced higher levels of downstream signaling than a bivalent design (Tao et al. 2019). This suggests that to reach maximal signaling activity, both FZD homodimerization and FZD-LRP5/6 heterodimerization are required. Although the exact size and stoichiometry remains to be determined, it has been suggested that WNT-FZD-LRP5/6 complexes can form even larger multimers, as visualized *in vivo* in both frog (Mii et al. 2017) and zebrafish (Hagemann et al. 2014).

3.1.2 DVL Recruitment

Following WNT-FZD-LRP5/6 binding, it is DVL that ultimately mediates inactivation of the destruction complex. Mammals express three DVL paralogs (DVL1/2/3), which have partially redundant functions *in vivo* (Etheridge et al. 2008; Lee et al. 2008; Gentzel and Schambony 2017; Ngo et al. 2020).

DVL shares several features with AXIN, including its “beads on a string” structure (Bienz 2014). The main structured domains in DVLs are the homo- and hetero-oligomerization DIX (highly similar to the DAX domain found in AXIN), PDZ (an acronym of post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1) and zonula occludens-1 protein (zo-1)), and DEP (Dishevelled, Egl-10, and Pleckstrin domain) domains, which are connected by flexible, unstructured linkers. The PDZ and the DEP domains have been shown to bind to FZD (Umbhauer 2000; Wong et al. 2003; Cong et al. 2004; Tauriello et al. 2012; Gammons et al. 2016a). This binding already occurs in the absence of signaling, but WNT proteins induce additional recruitment of DVL to the receptor complex (Fig. 2a–c). Specifically, WNT-FZD binding induces the so-called DEP domain swapping, in which two neighboring DVL molecules exchange beta-strands to form a highly stable dimer (Gammons et al. 2016a). Subsequently, the DIX domains facilitate further oligomerization, generating high local concentrations of DVL at the plasma membrane (Schwarz-Romond et al. 2007a; Gammons et al. 2016a). The DIX domain is essential for DVL polymerization and, as such, plays a critical role in formation of the aforementioned signalosome, as well as for the interaction with the AXIN DAX domain (Schwarz-Romond et al. 2007a; Fiedler et al. 2011), which become important later.

Direct interaction of DVL with the receptor complex has been firmly established using biochemical approaches. More recently, DVL recruitment to the plasma membrane was also visualized under steady state conditions *in vivo* after low level overexpression in *Drosophila* embryos (Schaefer et al. 2018, 2020). Direct visualization of the dynamic, WNT-induced recruitment of endogenous DVL2 has also been achieved (Ma et al. 2020). As a large portion of DVL remains cytosolic, at least in cultured cells, the subtle translocation of endogenous DVL to the membrane is not easily visualized using conventional microscopy. This obstacle was recently overcome by using total internal reflection fluorescence (TIRF) microscopy, allowing a sole focus on the plasma membrane associated pool. WNT3A stimulation resulted in an increase of fluorescently-tagged DVL2 molecules with a longer retention time at the plasma membrane (Ma et al. 2020). Importantly, in the absence of WNT3A, DVL2 was mainly monomeric and WNT3A mainly induced dimers and trimers. The absolute number of DVL molecules in a single complex did not exceed ten. Another study, using a similar approach, did not find changes in DVL2 recruitment upon WNT3A stimulation, but detected similarly small DVL complexes of 1–10 molecules (Kan et al. 2020). Whether this is solely the result of FZD multimerization or whether additional processes play a role remains unknown. DVL has been shown to mediate further clustering of FZD-LRP5/6 heterodimers into larger signalosomes (Metcalf et al. 2010), but overall DVL oligomerization is likely to be modest. Regardless, FZD-DVL binding (through the DEP domain) and DVL oligomerization (through the DIX domain) are essential for downstream signaling (Gammons et al. 2016b). The main steps that follow include AXIN recruitment and LRP5/6 phosphorylation – but here the precise sequence of events becomes less clear.

3.1.3 LRP5/6 Phosphorylation

LRP5/6 contains multiple phosphorylation sites that have been reported to be phosphorylated by GSK3, as well as by different CSNK1 isoforms (Fig. 2d–e). Its cytoplasmic tail contains five PPP(S/T)P motifs with an adjacent x(S/T) site and two additional clusters surrounding the most C-terminal PPP(S/T)P motif. The PPP(S/T)P and x(S/T) motifs are phosphorylated by GSK3 and CSNK1A1/D/E, respectively (Mao et al. 2001; Tamai et al. 2004; Davidson et al. 2005; Zeng et al. 2005, 2007). GSK3 and CSNK1A1 are thought to be brought to the membrane along with AXIN (Zeng et al. 2007). Somewhat confusingly, while AXIN is required for LRP5/6 phosphorylation, LRP5/6 phosphorylation in turn is required for the LRP5/6-AXIN interaction (Mao et al. 2001; Davidson et al. 2005; Zeng et al. 2005). It could be that AXIN is first recruited by DVL (as elaborated below), which might indeed bring AXIN-bound GSK3 and CSNK1A1 in close proximity to the LRP5/6 tail, thereby allowing its phosphorylation and generating an AXIN binding site. GSK3 does not seem to require a priming phosphorylation to phosphorylate LRP5/6. Instead, GSK3 appears to prime LRP5/6 for CSNK1-mediated phosphorylation of the adjacent x(S/T) site, thus reversing the order of kinase activity compared to the destruction complex, where CSNK1A1 primes CTNNB1 for GSK3 (Zeng et al. 2005). Both the PPP(S/T)P and x(S/T) sites are important for propagation of the WNT signal, as mutations to either of these motives reduce CTNNB1 signaling output.

In stark contrast, another report suggested that the PPP(S/T)P motifs are not phosphorylated in response to WNT-FZD-LRP5/6 binding, but are already phosphorylated in the absence of WNT (Davidson et al. 2005). If and how these two opposing models can be reconciled is a question that even the science Twitter hivemind has failed to answer.

The two phosphorylation clusters surrounding the C-terminal PPP(S/T)P motifs are thought to be phosphorylated by CSNK1G in response to WNT. It was proposed that signalosome formation releases an auto-inhibitory confirmation of LRP5/6 such that the CSNK1G target sites becomes accessible (Bilic et al. 2007). Interestingly, the N-terminal, extracellular domain of LRP6 was shown to exist in different conformational states (Matoba et al. 2017). This report suggested that a bend in the extracellular domain blocks association of multiple LRP5/6 molecules, thereby preventing spontaneous phosphorylation and signalosome formation. Collectively, these data can be interpreted in light of a model in which the interaction of WNT with FZD-LRP5/6 causes straightening of the LRP5/6 molecule thereby also relieving auto-inhibition of the C-terminal tail. Consistent with the notion of such an auto-inhibitory model, LRP5/6 lacking its extracellular domains is constitutively phosphorylated and signaling competent (Mao et al. 2001; Tamai et al. 2004). Importantly, auto-inhibition does not only seem to concern CSNK1G-mediated phosphorylation, but also CSNK1A1- and GSK3-mediated phosphorylation, suggesting that this could be a central mechanism for LRP5/6 phosphorylation.

3.2 Inactivation of the Destruction Complex

Signalosome formation ultimately causes inactivation of the destruction complex. Different models have been described, as also reviewed elsewhere (Tortelote et al. 2017). Mechanistically, they involve the translocation, dissociation, internalization, saturation and changes in conformation of one or more components of the destruction complex. Because these processes are not necessarily mutually exclusive (Fig. 3a), we will discuss their relative contribution for each individual component of the destruction complex (Fig. 3b).

3.2.1 AXIN

Early work showed that WNT signaling reduces AXIN levels (Yamamoto et al. 1999; Willert et al. 1999; Tolwinski et al. 2003), which was thought to be a main mechanism of destruction complex downregulation. It was later shown, however, that CTNNB1 accumulation precedes AXIN1 downregulation, suggesting that a reduction in AXIN1 levels is unlikely to be the deciding event (Li et al. 2012). So how then is AXIN, the central scaffold of the destruction complex, affected upon pathway activation?

As alluded to in the preceding section, signalosome formation results in AXIN translocation to the plasma membrane. Here, it interacts with both DVL and the phosphorylated LRP5/6 tail. The AXIN-DVL interaction is mediated through the DVL DIX and the AXIN DAX domains (Cliffe et al. 2003; Bilic et al. 2007;

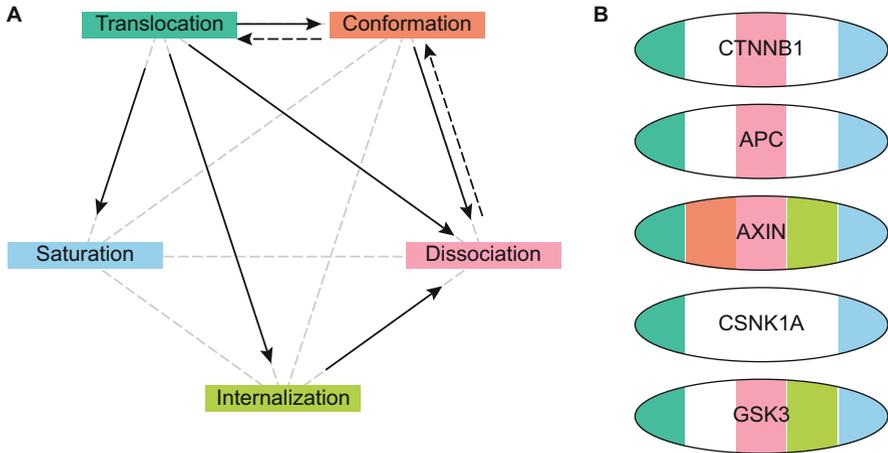


Fig. 3 Effects of WNT activation on destruction complex components. (a) Translocation, changes in Conformation, Dissociation, Internalization, and Saturation of different destruction complex components have all been shown to play a role in inactivation of the destruction complex. These processes are not necessarily mutually exclusive, and some events can precede others, as indicated by the arrows. (b) For the main destruction complex components and CTNNB1 we have indicated which of the processes depicted in (a) have been reported in the current literature

Schwarz-Romond et al. 2007b; Fiedler et al. 2011). The DIX-DAX interaction is weaker than either DIX-DIX or DAX-DAX interactions, and AXIN recruitment is thus thought to be facilitated by the local accumulation of DVL in the signalosome following WNT stimulation (Kan et al. 2020). Quantification of AXIN puncta at the plasma membrane in *Drosophila* suggested that tens to hundreds of AXIN molecules might be present in these spots (Schaefer et al. 2018). However, the structure of DIX oligomers suggests that a maximum of 4 AXIN molecules would be able to bind a single DVL oligomer (Kan et al. 2020). This discrepancy could be explained by the mild ($\sim 4\times$) overexpression of AXIN in the former study, or if signalosomes would contain multiple DVL oligomers, but could also reflect species specific differences.

The net result of AXIN recruitment to the receptor complex is thought to be an impaired destruction complex function, although there currently is little consensus on the exact mechanism (Fig. 3). First, as the DAX domain also facilitates AXIN self-oligomerization, which is necessary for destruction complex function, it was proposed that the DIX-DAX interaction reduces destruction complex activity by essentially exchanging AXIN-AXIN for AXIN-DVL interactions (Fiedler et al. 2011). Secondly, changes in AXIN phosphorylation and conformation, triggered at the activated receptor complex, have been described to reduce interactions with several of its binding partners, including CTNNB1 and GSK3 (Luo et al. 2007; Kim et al. 2013). All these changes are consistent with dissociation of the destruction complex and indeed many reports suggest (partial) disassembly of the complex upon WNT signaling. It should be noted, however, that others have shown that AXIN interactions with the core destruction complex components were unchanged at time

points up to 4 h after WNT stimulation (Li et al. 2012). This study instead argued that WNT binding leads to a loss of CTNNB1 ubiquitination, resulting in saturation of the intact destruction complex with CTNNB1 and accumulation of newly synthesized CTNNB1.

At first glance, the dissociation and saturation models are difficult to reconcile. The study that first proposed the saturation model looked at proteins that were pulled down and analyzed in bulk (Li et al. 2012). It is possible that not all destruction complexes dissociate, leaving some intact (Mukherjee et al. 2018). It has also been proposed that WNT stimulation leads to formation of large inactive destruction complexes in the cytoplasm, also called transducer complexes, which contain DVL as well as the standard destruction complex components (Hagemann et al. 2014; Lybrand et al. 2019). Without sufficient spatial and temporal resolution, therefore, the interactions of the total pool of AXIN might appear to remain relatively stable even if subfractions dynamically change composition. In the absence of a precise picture of the oligomerization status and protein stoichiometry of the different components, it is also unclear what saturation would look like for an individual destruction complex: Do both AXIN and APC bind CTNNB1, and how many binding sites in APC are expected to be occupied? And if the destruction complex is oligomerized, does every AXIN-APC module contain CTNNB1 or just some?

3.2.2 APC

APC translocation to the signalosome has also been shown to occur upon WNT pathway activation (Hendriksen et al. 2008; Habib et al. 2013). Like GSK3 and CSNK1A1, APC could be recruited together with AXIN (and thus be driven by DIX-DAX interactions). The dependence could also be reversed, however, as the recruitment of destruction complex components to a local source of WNT3A-loaded beads was shown to require full-length APC (Parker and Neufeld 2020). Although APC can bind microtubules (Munemitsu et al. 1994), thus potentially offering a mechanism for directional trafficking in the cell, how WNT binding would directly instruct APC relocalization to the signalosome remains unclear. That being said, intriguing links do exist between WNT/CTNNB1 signaling, mitotic cell division, and microtubule orientation and turnover (Salinas 2007; Stolz and Bastians 2015).

Recent data suggest that APC translocation to the signalosome might be short lived. Using a proximity-based labeling approach, the interaction of APC with the LRP5/6 signalosome was observed 5 min after WNT addition, only to be markedly reduced after 20 min (Colozza et al. 2020). If APC is indeed recruited through AXIN, loss of APC at the signalosome might represent dissociation of APC from AXIN, an observation that was made in many studies (Willert et al. 1999; Roberts et al. 2011; Valvezan et al. 2012; Tran and Polakis 2012; Ji et al. 2018; Mukherjee et al. 2018), except for that proposing the saturation model (Li et al. 2012). Loss of APC from the destruction complex might further stabilize CTNNB1, as this makes CTNNB1 prone to dephosphorylation by protein phosphatase 2A (PP2A) (Su et al. 2008). As APC is essential for destruction complex function, dissociation of APC could be sufficient to induce CTNNB1 stabilization and downstream signaling.

3.2.3 GSK3

It is safe to say that whereas GSK3 took center stage for some time, destruction complex inactivation is far more intricate than merely inhibiting GSK3 in the cytoplasm as initially suggested (Yost et al. 1998; Van Amerongen et al. 2005). While some studies have suggested that ubiquitination of CTNNB1, rather than phosphorylation, is inhibited in cells with active WNT signaling (Li et al. 2012; Azzolin et al. 2014), this is in direct contradiction with other experimental analyses and computational modelling efforts (Hernández et al. 2012; Kim et al. 2013). A recent study made an effort to directly compare these conflicting models of WNT-induced inhibition of CTNNB1 phosphorylation versus ubiquitination (Mukherjee et al. 2018). Combining biochemistry and modelling approaches, the authors ultimately concluded that both GSK3-mediated CTNNB1 phosphorylation and CTNNB1 ubiquitination are inhibited upon WNT signaling. If true, this work could settle a longstanding debate in the field, since it would suggest that both are required for the full dynamic range of WNT signaling. Assuming a critical role for GSK3 in this process, how does inhibition of GSK3 occur?

The simplest model describes that GSK3 is directly inhibited by LRP5/6 upon co-translocation to the signalosome together with AXIN (Cselenyi et al. 2008; Piao et al. 2008; Wu et al. 2009; Stamos et al. 2014) (Fig. 2d). As described above, here, GSK3 is thought to induce phosphorylation of the PPP(S/T)P motifs in the LRP5/6 tail. This phosphorylated motif provides a high-affinity pseudo-substrate for GSK3, leading to its sequestration and concomitant inhibition of GSK3 kinase activity (Piao et al. 2008). It was suggested that limited recruitment of four molecules of AXIN (and thus 4 GSK3 molecules) might provide an optimal stoichiometry for GSK3 inhibition, as each LRP5/6 tail contains a total of 5 PPP(S/T)P sites (Kan et al. 2020).

WNT activation has also been reported to lead to dissociation of AXIN-GSK3 interactions (Liu et al. 2005). More recently, however, bimolecular fluorescence complementation (BiFC) assays, which allow direct interaction rather than colocalization to be determined, have indicated that rather than a complete loss of interaction, the AXIN1-GSK3B complex adopts an alternative conformation that is not permissive to GSK3-mediated phosphorylation of CTNNB1 (Lybrand et al. 2019). If and how these findings reflect the LRP5/6 sequestration mechanism is unknown.

More than a decade ago, a substantially different model was proposed in which GSK3, attached to the receptor complex, leads to endocytosis-dependent internalization of the signalosome and subsequent sequestration of associated GSK3B (as well as AXIN and possibly additional destruction complex components) in multivesicular bodies (MVBs), insulating cytoplasmic CTNNB1 from phosphorylation and subsequent degradation (Taelman et al. 2010). Follow-up work has focused on the mechanisms responsible for GSK3B sequestration in MVBs (Dobrowolski et al. 2012; Vinyoles et al. 2014; Albrecht et al. 2018, 2020; Tejada-Muñoz et al. 2019; Colozza et al. 2020), as recently reviewed in detail (Colozza and Koo 2021). It has been proposed that macropinocytosis, a non-selective form of phagocytosis by which cells take up large amounts of extracellular fluid, is one of these mechanisms that ultimately trap GSK3 in MVBs (Tejada-Muñoz et al. 2019). Counter-intuitively,

these studies suggest that macropinocytosis is normally inhibited by AXIN1 and GSK3B, but that this inhibition is relieved when AXIN and GSK3 themselves become inhibited upon WNT signaling (Albrecht et al. 2020). If true, this prompts the question how macropinocytosis can be both the cause and the consequence of WNT-dependent GSK3 modulation. To add to the complexity, the role of endocytosis in WNT/CTNNB1 signaling is not fully resolved (Kikuchi et al. 2009; Yamamoto et al. 2017; Bandmann et al. 2019) and not without contention (Munthe et al. 2020; Rim et al. 2020), as extensively reviewed elsewhere (Brunt and Scholpp 2018; Wu et al. 2020; Colozza and Koo 2021).

3.2.4 CSNK1A1

Although CSNK1A1 has been shown to be essential for CTNNB1 degradation as the priming kinase for GSK3, if and how CSNK1A1 is inhibited upon WNT signaling has received little attention. Modelling studies from the Kirschner group suggested that CSNK1A1-mediated phosphorylation is inhibited upon WNT signaling (Hernández et al. 2012). However, this was not recapitulated in a more recent study (Mukherjee et al. 2018) and it is also not consistent with the notion of destruction complex saturation (Li et al. 2012).

WNT binding to FZD-LRP5/6 proteins can induce CSNK1A1 translocation to the plasma membrane in an AXIN-dependent manner (del Valle-Perez et al. 2011). This suggests that CSNK1A1 might be the predominant CSNK1 isoform that regulates LRP5/6 phosphorylation in the PPP(S/T)P-adjacent x(S/T) motifs. Further investigation of CSNK1A1 dynamics is warranted to reveal if and how CSNK1A1 location and function are regulated upon WNT signaling and to help resolve if and where CSNK1A1-mediated phosphorylation of CTNNB1 is impaired.

3.2.5 CTNNB1

In the end, impaired function of the destruction complex ultimately reduces CTNNB1 turnover. As discussed, what the exact contributions of inhibition of phosphorylation versus ubiquitination, or saturation versus (partial) dissociation are, will require further scrutiny. Our own recent work suggests that a substantial fraction of CTNNB1 remains bound to large complexes upon treatment with WNT3A, as measured by Fluorescence Correlation Spectroscopy (FCS) at endogenous levels (de Man et al. 2021). This is consistent with models where CTNNB1 remains part of an inactivated destruction complex (Li et al. 2012; Hagemann et al. 2014; Lybrand et al. 2019).

While attention mostly focuses on the cytoplasmic and nuclear accumulation of CTNNB1 in response to WNT stimulation, CTNNB1 translocation to the signalosome, presumably through AXIN, was revealed in E-cadherin knockout cells (Hendriksen et al. 2008). This pool of membrane localized CTNNB1 is usually masked by CTNNB1 associated with adherens junctions. Further understanding of the relevance of these different pools of CTNNB1 will require high spatiotemporal resolution and elucidation of complex composition and dynamics at physiological levels.

4 Targeting CTNNB1 and the Destruction Complex in Cancer

4.1 Hyperactivation of WNT/CTNNB1 Signaling

Dysregulation of WNT/CTNNB1 signaling occurs in many diseases, but most notably in cancer (Nusse and Clevers 2017). Mechanistically, mutations in destruction complex components and CTNNB1 itself are a common cause of oncogenic hyperactivation. Of these proteins, APC is most frequently mutated, followed by CTNNB1 and AXIN (Fig. 4a). Of note, mutations in different WNT/CTNNB1 pathway genes are predominant in different tumor types (Fig. 4b–d), highlighting the tissue-specific nature of genetic and epigenetic alterations in cancer (Schaefer and Serrano 2016; Schneider et al. 2017). For example, APC mutations are mainly associated with colon cancer (Fig. 4b), whereas CTNNB1 mutations are more prominent in liver and endometrial cancer (Fig. 4c).

The mutational spectrum of any given tumor is the result of a complex interplay between environmental factors (including mutagenic exposure) and molecular features of the cell involved – including the developmental origin of the tissue. As an example, it has long been known that different types of APC mutations result in different levels of WNT/CTNNB1 signaling activity (Albuquerque et al. 2002). While some of these offer a selective advantage to cells in the intestine, others can drive tumor formation in the mammary gland, a phenomenon coined as the “just right” level of signaling (Gaspar et al. 2009). This principle likely holds true even within different regions of the same tissue (Christie et al. 2013). As novel tools such as *in vivo* fluorescent reporters and single cell sequencing analyses become more readily available, we will be able to further determine the dosage effects of different mutations and their relation to tissue specificity. Given how subtle the differences in absolute levels and complex composition of the different WNT/CTNNB1 can be, understanding these subtleties will also be relevant in a pathophysiological context.

APC mutations typically occur in the so-called mutation cluster region (MCR) and cause protein truncation (Fig. 5a, b). Although these mutations remove some or all of the 20aa and SAMP repeats, truncated APC proteins retain some AXIN and CTNNB1 binding possibilities through their more N-terminal domains (Fig. 5c). Consistent with this, these truncated forms are able to downregulate CTNNB1 levels to some degree (Yang et al. 2006). This could partially be explained by the fact that even when CTNNB1 degradation is impaired, APC also has the capacity to retain CTNNB1 in the cytoplasm (Yang et al. 2006; Schneikert et al. 2007; Kohler et al. 2008; Li et al. 2012). Given all we know about the multivalent interactions in the destruction complex, however, it could also be that some level of CTNNB1 turnover is preserved. Indeed, depending on the mutation in the first APC allele, specific patterns of loss of heterozygosity (LOH) are observed for the second allele (Albuquerque et al. 2002; Christie et al. 2013).

CTNNB1 mutations also occur in a defined region of CTNNB1, namely the N-terminus (Fig. 5d–f). Specifically, CTNNB1 mutations that delete or alter N-terminal phosphorylation by CSNK1A1 (S45) or GSK3 (T41, S37, S33) or BTRC binding (S37-D32) occur frequently (Polakis 2000). Although all these

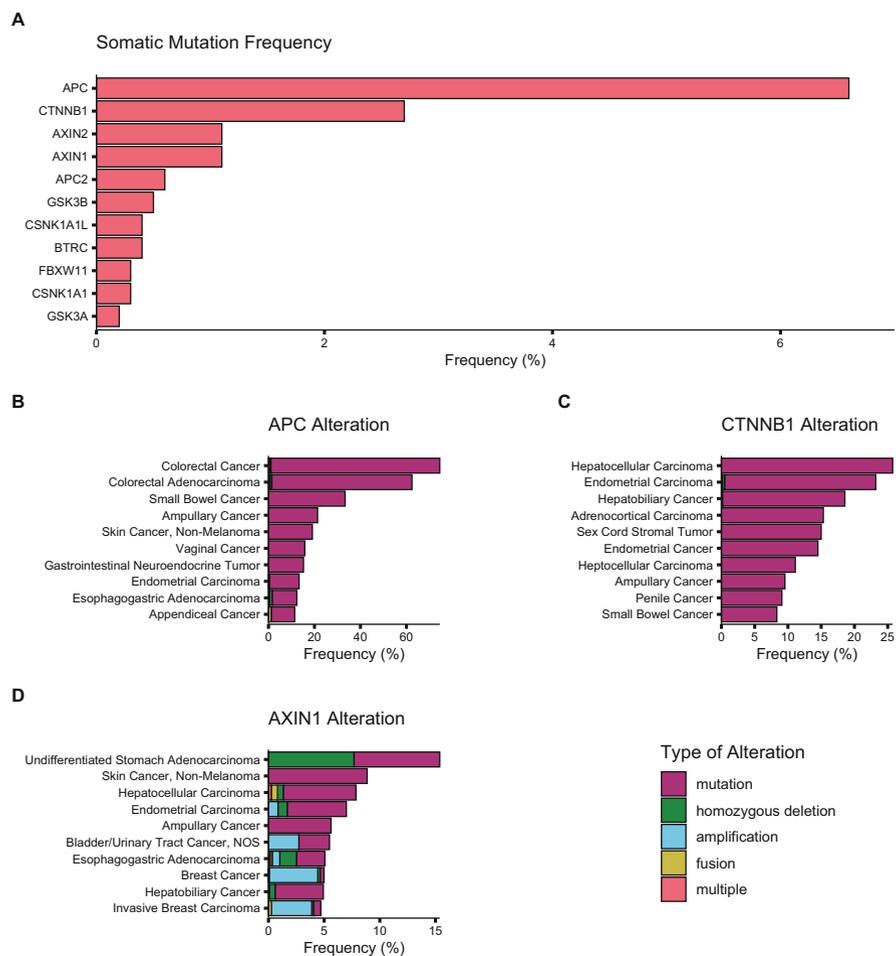


Fig. 4 Mutation of destruction complex components. Data were obtained from a curated set non-redundant studies through cBioPortal (Cerami et al. 2012; Gao et al. 2013) downloaded on the 2nd of February 2021. (a) Frequency of somatic mutations in destruction complex components, including homologs, and CTNNB1. FBXW11 is a close homolog of BTRC. (b–d) Alteration frequency of APC (b) CTNNB1 (c) and AXIN1 (d) in the top-ten predominant cancer types for those genes. Types of alterations legend in the bottom right corner

mutations are expected to render CTNNB1 unsusceptible to proteasomal degradation, there is some indication that both the position and specific amino-acid change could influence signaling potential (Austinat et al. 2008) and, similar to the situation observed for APC, could thus have different effects on the final signaling output of the WNT/CTNNB1 pathway.

Alterations to the *AXIN1* gene are less common in cancer (Fig. 4a), but have also been implicated in oncogenic WNT/CTNNB1 signaling (Bugter et al. 2020). The

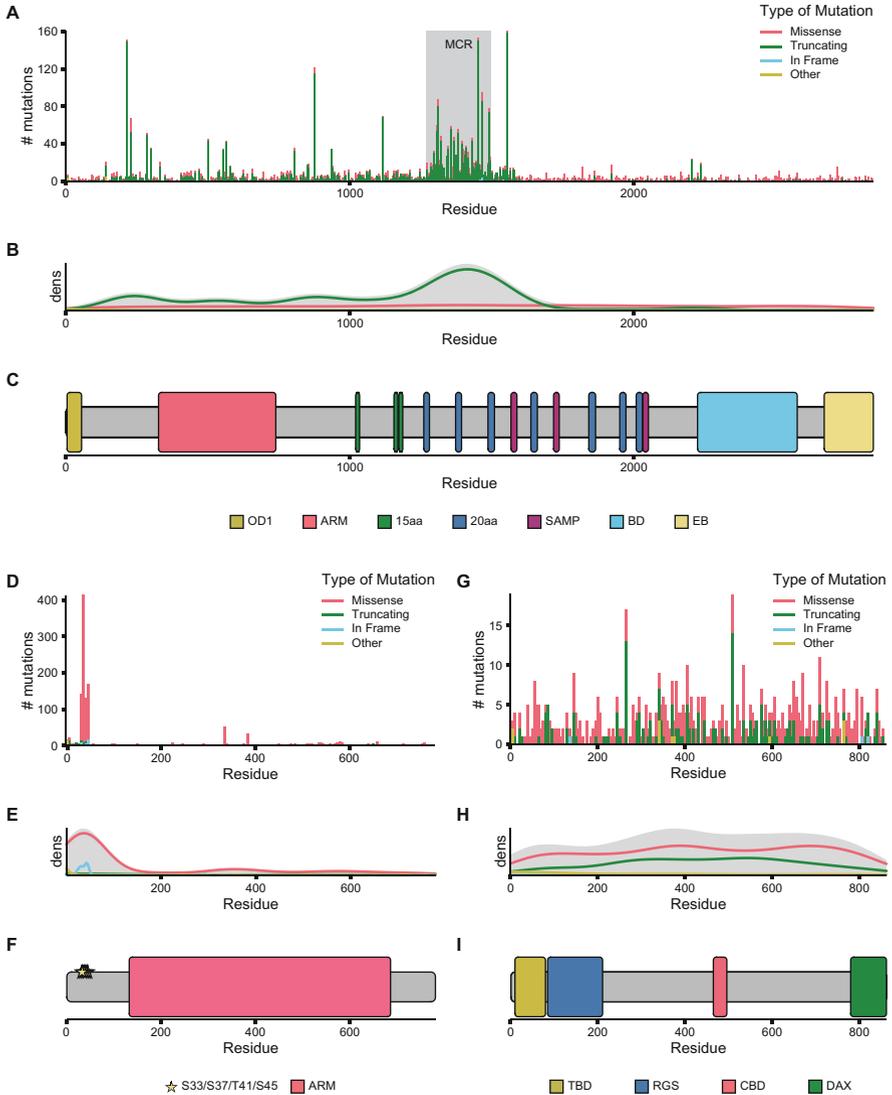


Fig. 5 Mutation data (**a, b, d, e, g, h**) were obtained from a curated set non-redundant studies through cBioPortal (Cerami et al. 2012; Gao et al. 2013) downloaded on the 2nd of February 2021. Domain information (**c, f, i**) was downloaded from Interpro (Blum et al. 2021) on the 4th of March 2021. Note that several domains (for example, the ASAD domain in APC) are not defined in Interpro and therefore not depicted. (**a, b**) Distribution of mutations in APC plotted as a histogram (**a**), or density distribution (**b**). (**c**) Domains of APC. OD1 = Oligomerization Domain 1, ARM = Armadillo repeats, 15&20aa = 15 and 20 amino-acid repeats, SAMP = Ser Ala Met Pro motifs, BD=Basic Domain, EB = EB-1 binding domain. (**d, e**) Distribution of mutations in CTNNB1 plotted as a histogram (**d**), or density distribution (**e**). (**f**) Domains of CTNNB1 and location of CSNK1A1 and GSK3 phosphorylation sites. ARM = Armadillo repeats. G-H) Distribution of mutations in AXIN1 plotted as a histogram (**d**), or density distribution (**e**). (**f**) Domains of AXIN. *TBD* Tankyrase Binding Domain, *RGS* Regulator of G protein Signaling domain, *CBD* CTNNB1 Binding Domain, *DAX* AXIN DIX

<u>Process</u>	<u>Hyperactivation</u>	<u>Therapeutic intervention</u>	
WNT-FZD-LRP5/6 association	FZD/WNT amplification WNT overexpression RNF43/ZNRF3 mutation	PORCN inhibitors soluble FZDs FZD blocking Abs	<i>Extracellular</i>
Signalosome formation	LRP5/6 hyperphosphorylation	CK1D/E inhibitors DVL inhibitors endocytosis / V-ATPase inhibitors ?	<i>Cytoplasmic</i>
Destruction complex inactivation	APC mutation AXIN mutation	Tankyrase inhibitors CSNK1A1 activators AXIN2 oligomerization peptide Endocytosis inhibitors ?	
CTNNB1 accumulation	CTNNB1 mutation	PROTACs	<i>Cytoplasmic</i>
CTNNB1-mediated transcription	BCL9 overexpression	TCF inhibitors BCL9 inhibitors	<i>Nuclear</i>

Fig. 6 Therapeutic targeting at multiple levels of WNT/CTNNB1 signaling

most predominant changes to *AXIN1* are genetic mutations or deep deletion of both *AXIN1* alleles. Compared to *APC* and *CTNNB1*, *AXIN1* mutations are much more spread throughout the gene body, without a clear hotspot (Fig. 5g-i). In-depth analysis of mutations located in the RGS domain showed that these variants form nanoaggregates through the mutated RGS domain, which resulted in a change in the AXIN interactome, thereby perturbing normal destruction complex assembly (Anvarian et al. 2016). However, the consequence of most other *AXIN* mutations remains to be uncovered.

In addition to genetic alterations in destruction complex components and CTNNB1, other mechanisms of hyperactivation occur in cancer (Fig. 6), as also reviewed elsewhere (Wiese et al. 2018; Bugter et al. 2020). For example, FZD protein levels can be elevated through amplification, overexpression or mutations in negative regulators such as E3 ubiquitin-protein ligase RNF43 and E3 ubiquitin-protein ligase ZNRF3 (Koo et al. 2012; Giannakis et al. 2014). Although these alterations occur at different levels of the pathway, they share the consequence of CTNNB1 stabilization and, as such, are targets for therapeutic intervention.

4.2 Therapeutic Targeting

Historically, the first focus was on direct inhibition of CTNNB1 transcriptional activity. Many drugs targeting specific interactions between CTNNB1 and TCF or other parts of the enhanceosome have been developed (Wang et al. 2021b). Although new compounds continue to be discovered, few of these drugs have

progressed to clinical trials – a fate that is not uncommon for interventions aimed at targeting transcription factors in the nucleus (Darnell 2002; Chen and Koehler 2020). Especially in the last decade, attention has shifted to inhibiting WNT/CTNNB1 signaling at the membrane. For example, Protein-serine O-palmitoleoyltransferase porcupine (PORCN) inhibitors, which block the palmitoylation of WNT proteins and should thus impair both WNT secretion and FZD binding, are currently in clinical trials (Shah et al. 2021). Soluble decoy FZDs and FZD antibodies have also been deployed to catch away the WNT or block WNT binding, with ipafricept (or OMP-54F28) completing Phase I trials (DeAlmeida et al. 2007; Jimeno et al. 2017; Moore et al. 2019) and vantictumab (or OMP-18R5) showing promising clinical activity in a breast cancer cohort (Gurney et al. 2012; Diamond et al. 2020). It is not our goal to provide a comprehensive overview of all prior and ongoing efforts, however. For this, we refer the reader to some excellent recent reviews on this topic (Zhan et al. 2017; Krishnamurthy and Kurzrock 2018; Blagodatski et al. 2020; Jung and Park 2020; Wang et al. 2021b).

As it turns out, even cancer cells with APC mutations have been reported to respond to WNT stimulation in some cases, and might therefore also be sensitive to pharmacological inhibition at the receptor level (Suzuki et al. 2004; Voloshanenko et al. 2013; Flanagan et al. 2019). While this may not seem logical at first, it suggests that a better understanding of the destruction complex and its inactivation could offer a window of opportunity to target the WNT/CTNNB1 pathway in the cytoplasm. So where would these drugs come from and which components would we target (Fig. 6)?

4.2.1 Natural Compounds

Over the years, a host of natural compounds has been reported to modulate WNT signaling activity. For many of these, the precise mode of action remains unclear. In the case of WNT/CTNNB1 inhibitors, the capacity to modulate CTNNB1 levels, to reduce target gene expression, to block cell proliferation or to induce apoptosis are often the only readouts used (Fatima et al. 2017; Li et al. 2019). Some of these activities were discovered coincidentally, but other compounds were isolated in dedicated screens and, as such, are interesting leads to develop new therapeutics in the age of personalized medicine (Blagodatski et al. 2020), provided that the mechanism of action is ultimately resolved.

As an example, prodigiosin (a red pigment produced by different species of bacteria) has pleiotropic activities, including an inhibitory effect on WNT/CTNNB1 signaling. Mechanistically, prodigiosin has been shown to reduce the phosphorylation of both LRP6 and DVL (Wang et al. 2016). Due to its chemical structure, prodigiosin (as well as other natural compounds such as salinomycin and nigericin) could have the capacity to interfere with the activity of vacuolar H⁺-adenosine triphosphatase (V-ATPase), which would inhibit the acidification of small vesicles (Castillo-Ávila et al. 2005). If endocytosis indeed plays a critical role in either activation of the signalosome (by promoting LRP6 phosphorylation) or in inactivation of the destruction complex (Cruciat et al. 2010; Taelman et al. 2010), this could explain the mode of action for these drugs – which may be clinically relevant.

4.2.2 High-Throughput Chemical Screens

Screening of large chemical libraries has identified other compounds that could potentially reduce signalosome formation. For example, longdaysin was identified as a CSNK1 inhibitor in a screen for modulators of the circadian rhythm from a library of 120,000 compounds (Hirota et al. 2010). It was later shown to also inhibit WNT/CTNNB1 signaling, presumably by inhibiting CSNK1D- and CSNK1E-mediated LRP5/6 and DVL phosphorylation (Xiong et al. 2019). Novel developments in high throughput screening, such as virtual screening of molecules against resolved protein structures, promise to facilitate identification of novel compounds at reduced costs (Hori et al. 2018; Li et al. 2020). For example, virtual screening of >5,000 compounds against the DVL PDZ domain identified 1700 candidates for further screening, effectively reducing the need for costly experimental screens (Hori et al. 2018). Five of these DVL binding compounds were shown to have antiproliferative effects in a triple negative breast cancer cell line with active WNT/CTNNB1 signaling (Hori et al. 2018). Inhibition of signalosome formation could be particularly interesting in cases where FZD-LRP5/6 proteins are overexpressed (Yang et al. 2011; Ueno et al. 2013). Especially in those cases where LRP5/6 is involved, either as a result of overexpression or hyperphosphorylation, FZD blocking antibodies might in and by themselves not be sufficient to prevent hyperactivation of the WNT/CTNNB1 pathway (Raisch et al. 2019).

High-throughput screens have also revealed small molecules that can reactivate destruction complex function. For example, in a screen of FDA-approved drugs, pyrvinium, which was previously used to treat pinworm infections, was found to stimulate CSNK1A1-mediated phosphorylation of CTNNB1, thereby rescuing CTNNB1 degradation (Thorne et al. 2010). Structural modification or additional screens can be employed to find inhibitors with stronger specificity for individual isoforms or enhanced clinical properties. Compared to pyrvinium, for example, the second generation CSNK1A1 activator SSTC3 has enhanced bioavailability, showed low toxicity in mice and is able to cross the blood brain barrier – all properties that are highly relevant for clinical applicability (Li et al. 2017; Rodriguez-Blanco et al. 2019; Shen et al. 2020).

4.2.3 Rational Design

At the other end of the spectrum, drugs are being developed by rational design. For example, small molecules that bind CTNNB1 are now used to generate proteolysis targeting chimeras (PROTAC) (Jiang et al. 2018). These PROTACs include a small molecule domain that recruits E3 ubiquitin ligases, thereby restoring CTNNB1 degradation even in the absence of destruction complex activity. A known CTNNB1-binding peptide derived from AXIN coupled to a PROTAC degradation tag was able to durably reduce CTNNB1 levels in organoids and mouse models carrying APC mutations (Dietrich et al. 2017; Liao et al. 2020). In another approach, an allosteric site of CTNNB1 that does not bind to any known CTNNB1 partners was discovered (Cheltsov et al. 2020). Subsequent screening for specific binders of the allosteric site identified a molecule CS2 that was also able to target CTNNB1 for

degradation. Because CTNNB1 has multiple interaction partners in the cell, for which the binding sites largely overlap (van der Wal and van Amerongen 2020), using this allosteric site allows targeting of CTNNB1 without disrupting binding events that are important for normal cell function, such as the interaction with E-cadherin in the adherens junction.

For many of these natural compounds, small molecules, and designer drugs it is early days, of course, and only time will tell whether they will progress to clinical trials. However, one targeting approach that aims to reinstate destruction complex activity through inhibition of Poly [ADP-ribose] polymerase tankyrases (TNKS1/2) has been in the pipeline for some time and shows promising results. TNKS1/2 normally targets AXIN for PARsylation, which in turn leads to AXIN degradation. In 2009, a first generation tankyrase inhibitor (XAV939) was discovered in a chemical screen using a CTNNB1/TCF reporter gene readout (Huang et al. 2009). Some TNKS inhibitors have now progressed to clinical trials (Mehta and Bhatt 2021). For example, TNKS inhibitor E7449 is currently in Phase II trials, after showing promising results in Phase I, where a gene expression matrix was used to predict drug response (Plummer et al. 2020). Interestingly, boosting AXIN levels is sufficient to restore destruction complex activity, even in the presence of APC truncations (Nakamura et al. 1998). Here, the position of the truncation, and thus the degree to which APC retains the capacity to interaction with AXIN and CTNNB1 determines the response to tankyrase inhibition (Tanaka et al. 2017; Schatoff et al. 2019). The ability to predict drug responses based on specific mutations or expression profiles brings us one step closer to realizing the promise of personalized medicine.

5 Summarizing Conclusion

As we have hopefully illustrated, a more detailed, quantitative understanding of the WNT/CTNNB1 pathway is helping us to fully resolve the molecular mechanisms that underlie signal transduction. This will not only create new opportunities for therapeutic intervention but will also help overcome the challenges involved – because in the end, it is this complex and dynamic system that we need to target. A better notion of which components are rate limiting enhanced insight into destruction complex composition and a firm grasp on overall protein stoichiometry at critical regulatory steps will allow us to build predictive models for how cells will respond to either the presence of specific mutations or to specific inhibitors. In the future, such models could identify which patients would benefit from which treatment. In the shorter run, they may reveal new critical nodes and perhaps counterintuitive mechanisms for drug-mediated intervention.

For starters, these studies show that longstanding dogmas in the field are in need of an overhaul. As an example, the notion that APC, and not AXIN, might be rate limiting for destruction complex formation or function has not yet been commonly adopted in the literature. We also want to highlight the familiar view that receptor complex activation by WNT proteins, and subsequent inactivation of the destruction

complex, allows CTNNB1 to accumulate in the cytoplasm and to translocate to the nucleus. Those working in the field know that the overall rise in CTNNB1 levels in response to physiological levels of WNT signaling is typically modest and it has been suggested that fold-change rather than absolute levels of CTNNB1 instruct signaling output (Goentoro and Kirschner 2009). Nuclear enrichment of CTNNB1 is also far from black and white in many circumstances – even in tumors. Yet, somehow, this subtle change in absolute levels in combination with mild nuclear enrichment is sufficient to result in the robust activation of tissue-specific target genes with major phenotypic consequences. A recurrent finding from these quantitative analyses is that the measured biological changes are small and subtle, reaching two- to four-fold at most (Jacobsen et al. 2016; Kafri et al. 2016; Massey et al. 2019; de Man et al. 2021) and often involving tens to hundreds of molecules.

Many questions remain, of course. First, several processes downstream of CTNNB1 stabilization contribute to the transcriptional outcome. Changes in nuclear shuttling – switching from nuclear exclusion to nuclear enrichment – and increased nuclear retention through binding to the TCF/LEF transcriptional complex also appear to be crucial for the final output of the pathway (Krieghoff et al. 2006; Kafri et al. 2016; de Man et al. 2021). Regulation of CTNNB1 nuclear shuttling and retention remain incompletely understood, but changes in CTNNB1 post-translation modifications and interactions with binding partners have been shown to play an important role (Gottardi and Gumbiner 2004; Hendriksen et al. 2008; Wu et al. 2008; Sayat et al. 2008; Valenta et al. 2012; van der Wal and van Amerongen 2020). Therefore, further elucidation of these processes might provide additional therapeutic targets in the future, as exemplified by a putative role for the transcriptional co-activators B-cell CLL/lymphoma 9, and 9-like protein (BCL9/BCL9L) in triple negative breast cancer (Wang et al. 2021a). In addition, novel regulators of the pathway continue to be discovered, for example through genetic screening (Lebensohn et al. 2016; Steinhart et al. 2016). As long as these discoveries continue to go hand in hand with an increased fundamental understanding of the molecular mechanisms that control WNT/CTNNB1 signaling, we will ultimately be able to use this knowledge to gain novel possibilities for therapeutic targeting or to predict which patients benefit from which drugs.

Second, a note on redundancy, which we have largely ignored so far. All four destruction complex components have one or several paralogues, which are also implicated in cancer to some extent (Fig. 4a). For example, GSK3A and GSK3B, encoded by different genes, have been shown to be redundant in phosphorylating CTNNB1 (Doble et al. 2007). For CSNK1, however, it is thought that mainly casein kinase alpha (CSNK1A1) is important for priming CTNNB1 in the destruction complex, despite the fact that multiple isoforms exist (Stamos and Weis 2013; Lebensohn et al. 2016). APC2, slightly smaller than APC itself, is often used in experimental studies from a practical perspective. Although less efficient in targeting CTNNB1 for degradation, it has some redundancy with APC in specific contexts, especially when APC is truncated (Schneikert et al. 2013; Daly et al. 2017). AXIN also comes in two flavors. Whereas *AXIN1* is ubiquitously expressed, the *AXIN2* gene is a universal and direct WNT/CTNNB1 target providing a negative feedback

loop (Jho et al. 2002; Lustig et al. 2002). Both AXIN1 and AXIN2 are mutated in cancer (Bugter et al. 2020), and also functionally redundant to some extent (Chia and Costantini 2005; Lebensohn et al. 2016). The finding that AXIN2 oligomerization is distinct from AXIN1 has led to the development of a short peptide that enhances AXIN2 polymerization and represses the growth of colorectal cancer cells (Bernkopf et al. 2015, 2019). Altogether, the functional and tissue-specific differences in expression between these homologous genes are barely understood or exploited and are worthy of follow-up.

Third, destruction complex identity and dynamics remain somewhat elusive. The high mobility and multivalency, as well as the large proportions of disordered regions in APC and AXIN, have led to the proposition that in the intracellular environment the destruction complex actually forms large, phase separated structures, also termed biomolecular condensates (Schaefer and Peifer 2019). Indeed, a recent study showed that AXIN is able to undergo liquid-like phase separation through its intrinsically disordered region *in vitro* (Nong et al. 2021). Phase separation might help explain how relatively low affinity interactions can lead to an efficient degradation machine. In this respect, the destruction complex might best be compared to Velcro, with multiple tiny interactions (and in the case of the destruction complex also multiple different ones) providing strength in association. This also means that any of these interactions could be tweaked to modulate destruction complex function as discussed in this review. And, in fact, it could also very well be that a one-size-fits-all definition of “the” destruction complex does not exist. Perhaps we should let go of the desire to arrive at a grand unifying model and instead appreciate the variation that exists across different cell types.

The combination of super resolution microscopy and other image-based techniques holds great promise for determining the shapes and sizes of different protein complexes as well as for resolving the different interactions between the individual components. For instance, endogenous DVL2 oligomerization at the cell membrane (at most ten molecules) as measured via TIRF microscopy (Kan et al. 2020; Ma et al. 2020) might be more subtle than anticipated based on prior studies using Fluorescence Correlation Spectroscopy and steric-exclusion chromatography studies, which estimated that WNT3A induced DVL3 supramolecular complexes of 40 MDa (~50 molecules) in the cytoplasm (Yokoyama et al. 2010, 2012). On the one hand, this emphasizes the need for subcellular resolution and measurements at endogenous levels. On the other hand, these discrepancies could also be due to a biological difference between DVL paralogs or experimental setups. Of course in real life, signal transduction is the net effect of a series of dynamic interactions. Here, the precise timing of the observation may have an impact on the perceived event, as illustrated by the aforementioned dissociation and consecutive reassociation of FZD dimers in response to WNT (Petersen et al. 2017). A similar situation might be at play when it comes to DVL: While WNT stimulation has been reported to induce mild recruitment of DVL to the plasma membrane (Ma et al. 2020), WNT-FZD binding has also been postulated to cause DEP domain dissociation from the receptor complex (Gammons et al. 2016a). While seemingly opposing and incompatible, we reason that an initial WNT-induced recruitment of DVL to the cell surface could

very well be followed by its subsequent dissociation to give rise to transducer complexes (Hagemann et al. 2014). This should be a testable hypothesis with today's live-cell imaging techniques.

Image based approaches can also be applied creatively in other contexts. As an example, artificial clustering of the LRP5/6 cytoplasmic tail is sufficient to induce downstream signaling, which was recently exploited to build an optogenetic switch to induce WNT/CTNNB1 signaling (Repina et al. 2019). To what extent clustering is a requirement per se under physiological conditions remains to be determined. As another example, solving the ligand/receptor specificity for the 19 WNTs and 10 FZDs has turned out to be a complex puzzle of which only some pieces have been put together using biochemical methods. Image based methodologies can determine binding affinities in living cells (Eubelen et al. 2018; Wesslowski et al. 2020; Eckert et al. 2020; Schihada et al. 2021) and as such offer promising new avenues of testing WNT/FZD specificity.

Regardless of the contexts they will be applied in, it can be expected that in the next decade structural, quantitative and dynamic studies such as the ones highlighted here will continue to increase our fundamental understanding of the processes that underlie WNT/CTNNB1 signaling and contribute to the development of tailored treatments.

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